EXHIBIT 6

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Short Report

Intrachromosomal triplications: molecular cytogenetic and clinical studies

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A newborn boy had meconium aspiration syndrome, hypospadias, a supernumerary digit on the left hand, hyperbilirubinemia, a fractured right clavicle, osteopenia, liver calcification, and mild pulmonary hyperplasia. Cytogenetic studies showed a chromosome 13 with additional material in 33% of the metaphases. The add(13) was considered to be a probable duplication of 13q12q22. The 13 paint probe hybridized to the add(13) from end to end. Fluorescence *in situ* hybridization (FISH) studies using retinoblastoma probe (RB)-1 that maps to 13q14 and D13S585 that maps to 13q32-q33 gave one signal for RB and three signals for D13S585. The pattern of the three signals from the 13q32q33 region and the G-banding pattern was best explained as a triplication of 13q22q33, with an inverted middle repeat resulting in tetrasomy for this segment. Mosaicism was confirmed by FISH using a D13S585 probe on a buccal smear. Three triplications detected in our laboratory were compared – 13q22q33, 15q11q13, and 2q11.2q21. FISH was critical in identifying triplications 13q22q33 and 15q11q13. The hybridization pattern also indicated an inverted middle repeat. We conclude that intrachromosomal triplications may be more prevalent than previously assumed and they probably share a common mechanism in their formation. When the G-bands do not correspond exactly to a duplication or to a tandem triplication, an important consideration is that the majority of triplications have an inverted middle repeat. Triplications can be mistaken for duplications. Therefore, in assessing duplications, FISH confirmation is recommended.

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Intrachromosomal triplications producing partial tetrasomy are rare; at least 16 patients have been described involving six chromosomes (2q37, 2q11.2q21, 5p14p15.33, 7p21.3p22, 9p13p22, 9p22pter, 10q26, 15q11q13 (8 cases), and 16q12.1q12.2) (Table 2). In this study, a novel mosaic triplication of 13q22q33 is reported and compared with other tetrasomies and trisomies for this region. The triplication 2q11.2q21 included in this study has been previously published (1). Triplications in the literature and those identified in our laboratory are reviewed to gain further insights into this rare abnormality.

Clinical report

A newborn male who weighed 3850 g was born after a 39-week gestation to a 15-year-old G1, P0, 0+ single white female. Pregnancy was complicated by *Chlamydia* infection, which was treated in early pregnancy, and smoking two packs of cigarettes per day. The delivery was normal. Apgar scores were 2 at 1 min, 6 at 5 min, and 7 at 10 min. Thick meconium-stained fluids were noted below the vocal cords in the delivery room. The patient required resuscitation and was on a mechanical ventilator. Chest X-ray was unremarkable for a bell shaped thorax and slightly dysplastic-appearing ribs. Lung fields were relatively clear and normal cardiac silhouette was noted. The patient's respiratory status improved over the first 24 h.

On initial physical examination the patient had moderate hypospadias, a supernumerary digit on the left hand (Fig. 1).

A skeletal survey showed osteopenic bone densities, a slightly dysmorphic-appearing skull with significant osteopenia and generalized thinning of the calvaria, and a fractured right clavicle (Fig. 2).

The heart and renal anatomy were normal by ultrasound scanning. However, multiple calcified lesions were noted within the liver (Fig. 3). The investigation for TORCH (acronym for toxoplasmosis, other infections, rubella, cytomegalovirus and Herpes simplex) infections was negative, a differential diagnosis for the liver lesions.

At 4 months, the patient (Fig. 4) had microcephaly, frontal bossing, with prominent fontanels and temporal bones. Hypertelorism, palpebral fissures that slant up, and epicanthic folds were observed. The nose was bulbous and the nasal bridge was broad and depressed. The ears were low set. He had a long philtrum, a thin upper lip, thick lower lip, and a small mandible. The neck was short, with extra folds of skin, and erythema was observed. The nipples were wide set.

Cytogenetic studies

Peripheral blood cultures were synchronized with methotrexate (amethopterin 0.05 μg/ml; # M6770, Sigma, St Louis, MO) for 17 h and released with thymidine (2.5 μg/ml; # T5018, Sigma) for 5.5 h. Following the addition of colcemid, the cultures were harvested. Chromosomes were GTG-banded.

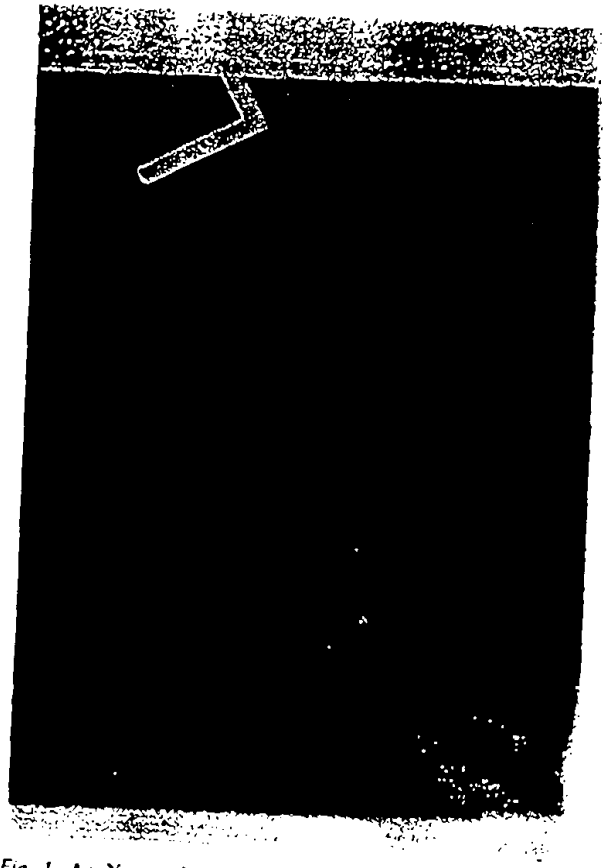

Fig. 1. An X-ray of the extra digit on the left hand.

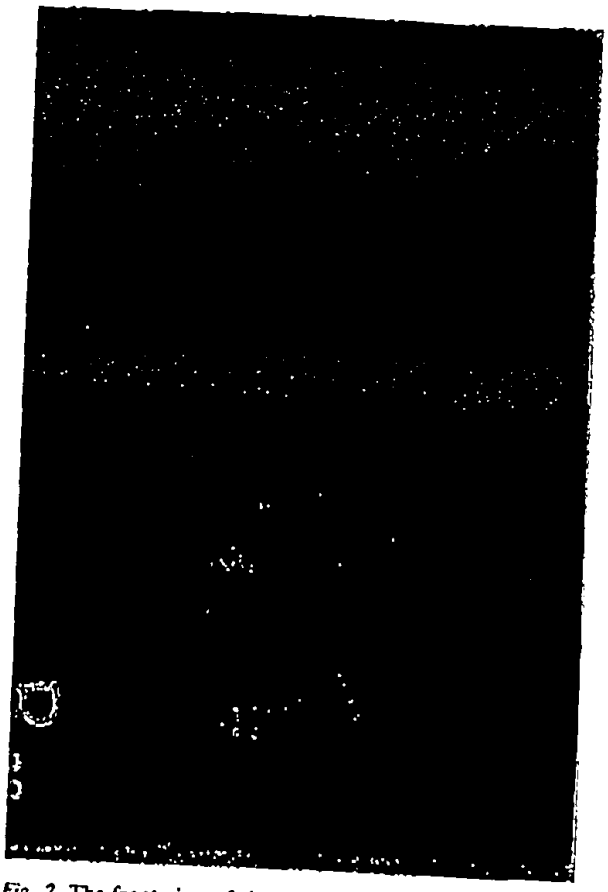

Fig. 2. The front view of the skull and the fractured clavicle.

Fluorescence *in situ* hybridization (FISH) studies were performed using the following probes: coatasome 13 and 2, retinoblastoma (RB) that maps to 13q14, D13S585 that maps to 13q32q33, and SNRPN, located in 15q11q13 (Oncor Inc., Gaithersburg, MD). The slides were denatured in 70% formamide/2 × SSC at 70°C for 2 min, followed by dehydration in 70, 80, and 90% absolute alcohol. Ten microliters of the probe were applied to the slide, coverslipped, and sealed. Following incubation overnight in a humid chamber at 37°C, the slides were washed in 1 × SSC (coatasome 13 and 2) or 2 × SSC (D13S585, RB-1, and SNRPN) at 72°C for 5 min. Detection was carried out using rhodamine-labeled anti-digoxigenin and counterstain 4,6-diamidino-2-phenylindole (DAPI).

An archived 1996 FISH slide of trp(15) was probed with D15S11 proximal to SNRPN on 15q11q13. The slide and probe were co-denatured at 85°C for 3 min. Following incubation overnight in a humid chamber at 37°C, the slides were washed in 2 × SSC at 72°C for 5 min. Detection was carried out using rhodamine-labeled anti-digoxigenin and counterstain DAPI.

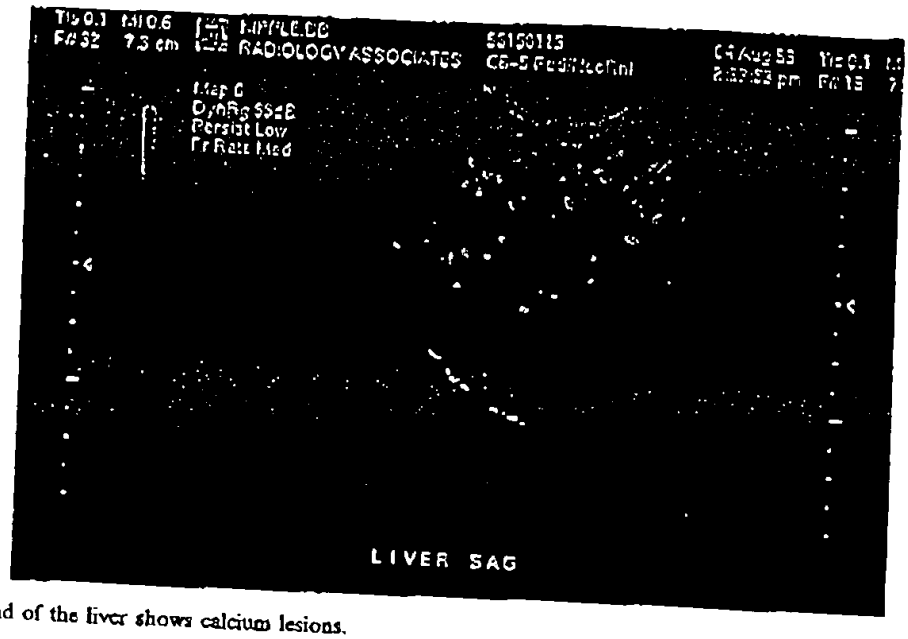

Fig. 3. Ultrasound of the liver shows calcium lesions.

The oral cavity of the patient was rinsed twice with drinking water. The inside of the oral cavity was scraped with a premoistened applicator or cytobrush. A smear was made by spreading the buccal mucosa uniformly across a prelabeled clean slide. The slides were fixed in ethanol before being transported to the laboratory. The quality of the slide was checked with a phase microscope for the presence of cells with adequate morphology. The slide was dipped in a coplin jar with 50% methanol and 50% acetic acid for 10 min. The air-dried slide was subject to 30% pretreatment solution (sodium bisulfite granular – Cat # 51337-1, Sigma) at 45°C for 15–20 min. Then the slide was rinsed for 1 min in 2 × SSC (pH 7) at room temperature and dehydrated in 70, 80 and 95 ethanol series for 2 min each at room temperature. Air-dried slides were checked for cell attachment. Ten microliters of the probe D13S585 were applied to the slide and co-denatured at 85°C for 7 min on a warm plate. The slide was hybridized overnight in a humid chamber at 37°C. Following a post-hybridization wash in 2 × SSC at 72°C for 5 min, detection was carried out according to the standard procedure.

Results

High-resolution analysis of G-banded chromosomes from lymphocyte cultures was performed. In case 1, a chromosome 13 duplication, ?dup(13)(q12q22), was suspected in 33% (7/21) of the metaphases analyzed (Table 1). A FISH study using whole chromosome painting probe (wcp) 13 painted both chromosomes 13 uniformly from end

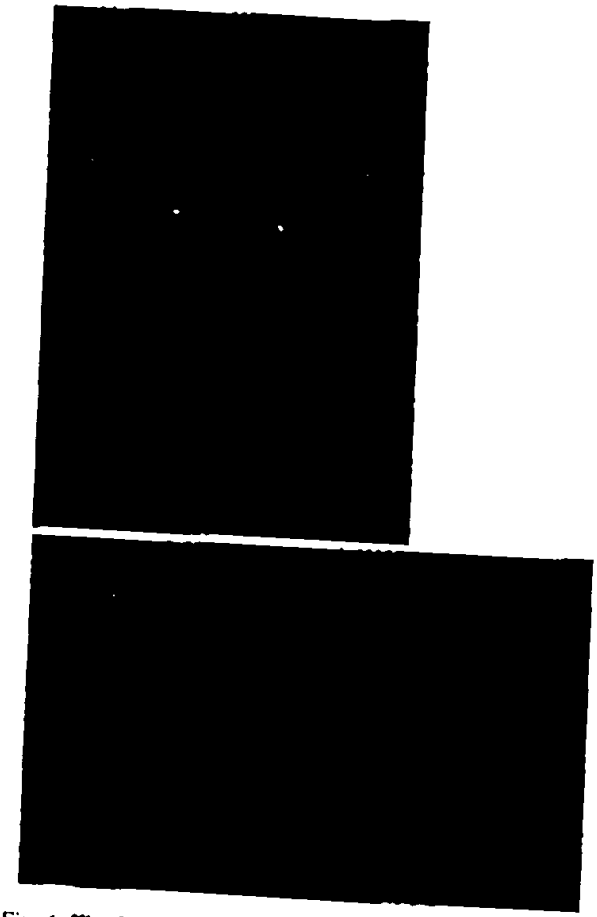

Fig. 4. The facial features of the patient at four months.

Table 1. Cytogenetic and FISH results from the three triplications in this study

Serial #	Age/sex	Indication	Karyotype
1	NB/M	Multiple congenital abnormalities	*de novo* mos 46,XY,?dup(13)(q12q22)[7]/46,XY [14]. ish trp(13)(q22q33)(wcp+, D13S585+++, RB-1+)
2	4/F	Microcephaly, developmental delay, and growth failure	*de novo* 46,XX,dup(15)(q11q13). ish trp(15)(SNRPN+++, D15S11+++)
3	Fetus/F	Brain malformations, multicystic kidneys, absence of the right thumb, and posterior cleft of palate	*de novo* 46,XX,trp(2)(q11.2q21). ish trp(2)(wcp2+)

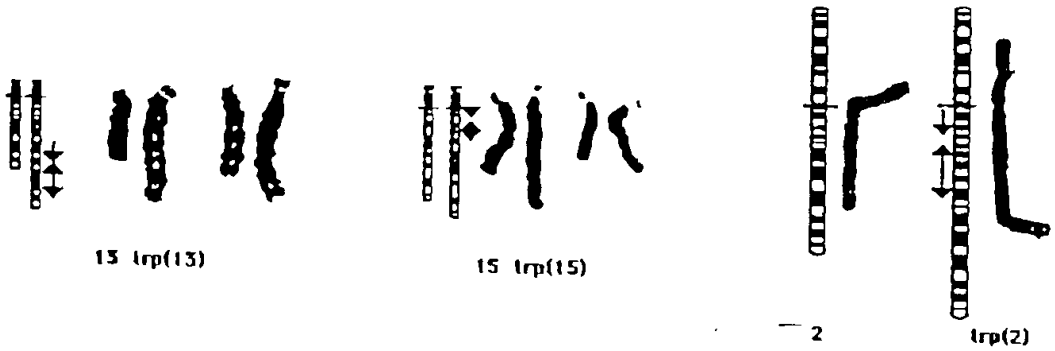

Fig. 5. Partial karyotype and ideogram for the triplications 13q22q33, 15q11q13, and 2q11.2q21 with the normal chromosome to the left and the chromosome with the intrachromosomal triplication on the right. The arrows show the triplicated segment and their orientation.

to end (Fig. 6A). The D13S585 probe gave two signals on the abnormal 13, the proximal signal was consistently larger than the distal signal (Fig. 6B). In interphases, 23% (45/200) had four signals (Fig. 6C) and two signals were frequently contiguous to each other. The RB probe gave a single signal on each of the two chromosomes 13 (Fig. 6D). The D13S585 pattern in interphases and the larger proximal signal on the abnormal chromosome 13 were an indication of an inverted middle repeat and of the distal position of this probe within the repeat (Fig. 8). The FISH finding suggested triplication of D13S585, and when collated with a G-banding pattern, triplication of 13q22q33 with an inverted middle repeat was apparent. Therefore, the karyotype was *de novo* mos 46,XY,?dup(13)(q12q22)[7]/46,XY [14]. ish trp(13)(q22q33) (wcp13+, D13S585+++, RB-1+). The mosaicism was confirmed in buccal smears using FISH and a D13S585 probe; 41% (18/44) of the interphases had four signals (Fig. 6E). Both parents' chromosome studies were normal in 50 metaphases studied. Therefore, the triplication is a *de novo* event.

Case 2 in G-banded metaphases was thought to have a duplication of 15q11q13 (Table 1, Fig. 5). FISH with a SNRPN probe gave three signals on one chromosome 15 (Fig. 7A). This confirmed a triplication of 15q11q13. The D15S11 probe that maps proximal to SNRPN gave two signals on a chromosome 15. The distal signal was consistently larger than the proximal signal (Fig. 7B). In interphases, the D15S11 probe gave four signals, and two signals were contiguous or very close to each other in the majority of cells (Fig. 7C). This strongly suggested a triplication with an inverted middle segment (Fig. 8).

The third case was a trp(2)(q11.2q21), which was identified in G-banding (Fig. 5). Coatasome 2 painted the triplicated chromosome from end to end.

Discussion

Mosaic tetrasomy for the terminal long arm of chromosome 13 has been reported previously (2–4) in the form of acentric markers. In this study, we describe the first mosaic intrachromosomal triplication of 13q22q33 and compare the phenotype to cases with duplication of 13q21qter. The trisomy 13q21qter clinical features (5) (from the Oxford Medical Database: Cytogenetics) found in our patient were frontal bossing, hypertelorism, palpebral fissures that slant up, bulbous nasal tip, small short nose, low-set ears, long philtrum, small mandible, thin upper lip, thick broad neck, and post-axial polydactyly of the fingers. Our patient shared many of the facial features with trisomy 13q21qter.

Table 2. Summary of reported cases with intrachromosomal triplications

Sl. #	Triplicated segment	Age	Middle repeat inverted	Other abnormalities	Parental chromosomes	Parental origin	Reference
1	2q37	15	–	No	Normal	–	Rauch et al., 1996 (13)
2	2q11.2q21	NB	Yes	No	Not tested	–	Weng et al., 1999 (1)
3	5p14p15.33	NB	Yes	No	Normal	Maternal (1)	Harrison et al., 1998 (14)
4	7p21.3p22	2	Yes	No	Normal	–	Rivera et al.,1996 (4)
5	9p13p22	8 mos	Yes	No	Normal	Paternal(2)	Verhelj et al., 1999 (15)
6	9p22pter	Infant	–	Idic(9)	Normal	–	Batanian et al., 1994 (16)
7	10q26	11	Yes	No	Normal	Maternal (2)	Devriendt et al., 1999 (17)
8	15q11q13	2 4/12	–	No	–	Maternal (2)	Holowinsky et al., 1993 (18)
9	15q11q13	7mos	–	No	–	Maternal (2)	Holowinsky et al., 1993 (18)
10	15q11q13	7	Yes	No	Normal	Maternal (2)	Schinzel et al., 1994 (19)
11	15q11q13	40	Yes	No	–	–	Crawford et al., 1995 (20)
12	15q11q13	17	–	No	–	Maternal (–)	Chadwick et al., 1996 (21)
13	15q11q13	6	–	No	–	Paternal (2)	Cassidy et al., 1996 (22)
14	15q11q13	4	Yes	Inv dup(15)	Normal	Maternal (2)	Long et al., 1998 (23)
15	15q11q13	4	Yes	No	Normal	–	Present study
16	16q12.1q12.2	3	–	–	Normal	Paternal(–)	Berry et al.,1990 (24)
17	13q22q33	NB	Yes	–	Normal	–	Present study

'–' indicates information that was not available. Age: NB, newborn; mos, months. Case 5 was reported as inversion triplication without mentioning the repeat that was inverted. Parental origin with 2 indicates that both maternal or paternal alleles were involved in the triplication, while 1 indicates that only one maternal or paternal allele was involved.

Liver calcification observed in 1/1750 fetuses (6) has been associated with prenatal viral infections, tumor or vascular embolism, hemangiomata, or vascular thrombosis (6). Such calcification has been observed in cases with chromosome abnormalities such as monosomy X, trisomies 9, 13, 18, 21 and partial trisomies 8 and 14 (7–12). Our patient was investigated for TORCH infections and found to be negative; therefore, the probable cause for the hepatic lesions is vascular insufficiency.

The chromosome region 15q11q13 appears to be prone to triplication since it is involved in 50% of

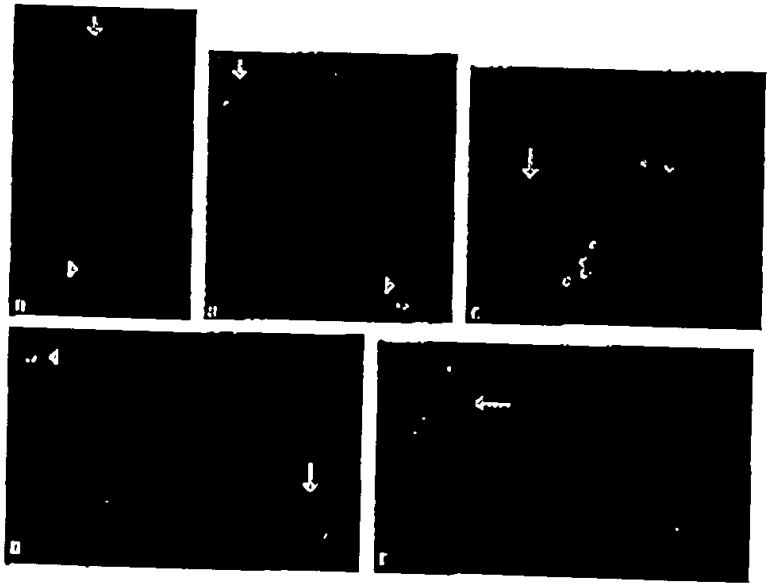

Fig. 6. The 13q22q33 triplication was ascertained using FISH. A) wcp 13 painted the normal chromosome 13 (arrowhead) and trp(13q22q33) (arrow) from end to end. B) The D13S585 that maps to 13q32q33 gave a large proximal and small distal signal (arrow) on trp(13q22q33) and a single signal on the normal 13 (arrowhead). C) In a majority of interphases, there were a total of four signals with two contiguous signals (arrow). D) The RB probe that maps to 13q14 gave a single signal on the abnormal 13 (arrow) and the normal 13 (arrowhead) and hence was not involved in the triplication. E) The mosaicism was confirmed in a buccal smear using a D13S585 probe. Interphases had four signals and two were contiguous or close to each other (arrow).

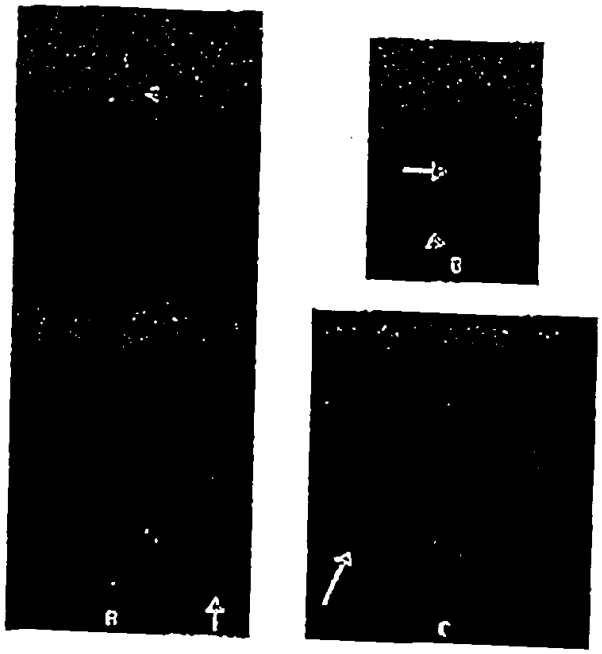

Fig. 7. FISH ascertainment of trp(15)(q11q13): A) SNRPN probe gave three signals on the abnormal 15 (arrow) and a single signal on the normal 15 (arrowhead). B) D15S11 probe gave a small proximal and a large distal signal on the abnormal 15 (arrow) and a single signal on the normal 15 (arrowhead). C) In interphases, there were three signals for D15S11 and two were contiguous to each other (arrow).

the reported cases (Table 2) (1, 3, 13–24). It is possible that the same hot spots involved in small inversion duplications and deletions of 15q11q13 (25) may also be involved in the triplications. A majority of triplications have an inverted middle repeat, suggesting a common mechanism of origin (Table 2). Seven triplications were on the maternally inherited chromosome and two on the paternal chromosome. The long prophase I of oogenesis may be the cause for the maternal triplication.

The mechanisms postulated in the formation of triplication were reviewed by Wang et al. (1999) (1). In summary, Schinzel et al. (1994) (19) suggested that intrachromosomal triplications arise from a U-type exchange resulting in a dicentric inverted duplication of 15pterq31, which occurs fairly frequently and is usually maternal in origin. The inv dup(15) breaks and recombines with a normal chromosome 15 to form the triplication. This is a two-step process. Alternatively, intrachromosomal triplications are compatible with U-type exchanges among three chromatids (1) in one step. Both these models can explain the concomitant occurrence of intrachromosomal triplication and a supernumerary dicentric marker seen in the case reported by Long et al. (1998) (23). In the two mechanisms outlined, the DNA polymorphisms

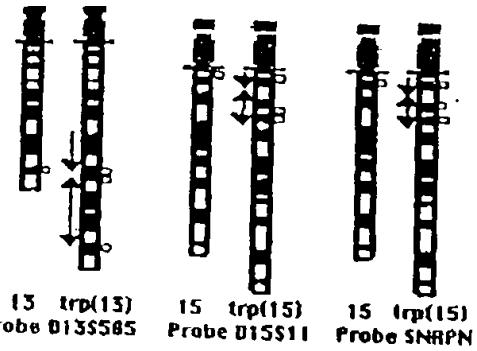

Fig. 8. The ideogram for a normal and triplicated 13q22q33 and 15q11q13, and the signal pattern gave an indication of an inversion of the middle repeat (shown by the direction of the arrows). A) The D13S585 locus when in the distal region of the triplicated segment and the middle repeat is inverted; the pattern of hybridization on chromosome 13 would be a larger proximal signal (or two contiguous signals in interphases) and a smaller distal signal. B) The D15S11 locus when in the proximal region of the triplicated segment and the middle repeat is inverted; the pattern of hybridization would be a smaller proximal signal and a larger distal signal on chromosome 15 or two contiguous signals in interphases.

would be from both homologs, but Harrison et al. (1998) (14) found polymorphisms derived from a single chromosome, suggesting yet another mechanism. The mosaic 13q21q33 triplication found in this study is most probably a mitotic event. If the triplication was meiotic in origin, the subsequent formation of the normal cells by the loss of the triplicated 13 and the nondisjunction of the normal 13 would result in both chromosomes 13 having identical polymorphism. Since that is not the case (Fig. 9), a mitotic event is the most likely explanation.

Among the three triplications – 2q11.2q21, 15q11q13, and 13q22q33 – detected in our laboratory, only triplication 2q11.2q21 was discerned in

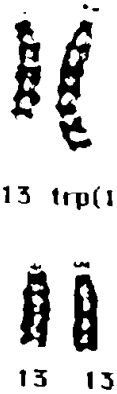

Fig. 9. Comparison of the polymorphisms on chromosomes 13 in the normal and trp(13) cells.

G-banded metaphases because of the size and distinct banding pattern of the repeat (1). Both subtle triplications, 15q11q13 and13q22q33, were thought to be duplications by G-bands and resolved to be triplications by FISH using SNRPN, D15S11, and D13S585 probes. Probes D13S585 and D15S11 gave two signals each: the proximal signal on 13 and distal signal on 15 were consistently larger in the majority of metaphases. However, in interphases, there were a total of four signals of equal size, and two signals in the majority of cells were contiguous (Fig. 6C Fig. 7C). This confirmed a triplication of D13S585 and D15S11. A larger proximal or distal signal on metaphases and two contiguous signals in interphases suggested a probable inverted middle repeat and a relative distal end position for D13S585 and proximal end position for D15S11 on the repeat (Fig. 8). If the probe is in the middle of the repeat, probably like SNRPN, the three signals would be spaced out. Although this probe identifies the triplication, it gives no information about the orientation of the repeats (Fig. 8). When the FISH data was correlated with the G-banding, it was a good fit for a triplication of 13q22q33 and 15q11q13 segments, with an inverted middle repeat. Some duplications by cytogenetic analysis may in fact be triplications and therefore warrant further investigation with FISH probes. The 15q11q13 duplications are not rare and cases not confirmed by FISH should be investigated using SNRPN and D15S11 probes. Since the majority of triplications (Table 2) have a middle inverted repeat, the signals may appear in metaphases as a larger extra signal which can be resolved into two separate signals in interphases except when the probe maps to a breakpoint. Further delineation of these segmental amplifications into duplications and triplications would provide a more accurate genotype-to-phenotype correlation.

The probe D13S585 is thought to map to 13q32q33. The FISH pattern observed for D13S585 suggests a distal position of the probe on the 13q22q33 repeat and narrows its mapped position to 13q33.

Acknowledgements

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